Capillary Electrophoresis

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Introduction

The migration of charged particles under the influence of an electric field was discovered and characterized theoretically more than 100 years ago by Kolrausch *et al*. Foreseeing the possibility of separation of charged species through the application of a voltage, the term 'electrophoresis' was coined soon after. However, early attempts to use electrophoresis as an analytical tool were persistently frustrated by the existence of Joule heating, which acts to discount the electrophoretic effect. Thus a way of combatting the thermal effect during the electrophoretic process was needed. By 1950s, Tiselius *et al*. found that a variety of substances such as agarose and polymeric gels could serve as stabilizing agents in electrophoretic analysis owing to their anticonvective properties. This eventually led to the creation of slab gel electrophoresis, which has become a fundamental technique for the study of proteins, DNA fragments and other biomacromolecules in life sciences and biotechnology. Notwithstanding its great success, slab gel electrophoresis has its drawbacks with respect to speed and automation when compared with contemporary chromatographic techniques such as high performance liquid chromatography (HPLC).

A straightforward way to speed up an electrophoretic separation process is to apply higher electric fields, and this necessitates systems able to release the heat generated more efficiently. Electrophoresis with a tube as a separation channel is hence an attractive choice since the desired surface-to-volume ratio can be achieved by simply reducing the tube radius. Performing electrophoresis based on the tube format has an added advantage in that simultaneous detection may be implemented in a way analogous to HPLC, thus rendering the entire procedure fast and automatic. Running electrophoresis with a tube configuration was initiated by Hjerten as early as the 1960s, and further attempted by Virtanen *et al*. and Mikkers *et al*. in 1970s. During this period, the adopted inner diameters of tubes were in the range of 0.2–3 mm, and thermal effects confined the applied voltage to around 1000–2000 V, which was of the same order as in typical slab gel electrophoresis. As a consequence, despite these pioneering efforts to perform free solution electrophoresis with in-line monitoring, the full potential with respect to column performance was not yet attained. Also, complexity in instrumental design deterred follow-up by ordinary electrophoresis practitioners.

A milestone for column-based electrophoresis was set in the early 1980s, when Jorgenson *et al*. introduced capillary zone electrophoresis (CZE) with on-column optical detection. They found that with the inner diameter of the capillaries scaled down to 80 μm, voltages as high as 30 kV could be applied without incurring overheating problems. Thus the separation time for most charged species, from small molecules to macromolecules, was shortened to less than 30 min, which is comparable to modern chromatographic methods. For the first time outstanding column efficiencies of several hundred thousand plates was routinely obtained. The unprecedented performance, relatively simple instrumentation, concurrent with the widespread availability of fused silica capillary columns by the mid-1980s quickly aroused the interests of both electrophoresis practitioners and chromatographers, thus making capillary electrophoresis (CE) one of the most exciting research areas. Today, it has become an indispensable branch of modern separation science. The powerful separation ability of CE was exemplified in an early electropherogram concerning the resolution of derivated peptides originated from egg white lysozyme (**Figure 1**).

This article serves as an introduction to CE. It covers the basic principles, various aspects of instrumentation, separation modes and major applications. Some future trends of CE are discussed in the final section.

Fundamentals

Electrophoretic Migration of Ions

The uniform motion of an ion under an electric field can be recognized as a result of balancing electromotive and frictional forces of the ion in solution:

$$qE = 6\pi r\eta u \qquad [1]$$

where q is the effective charge of the ion concerned, E is electric field, while r is the ion's Stokes radius, η is the dynamic viscosity of the solution, and u is the linear velocity of the ion. The important parameter, electrophoretic mobility (μ), is defined as the ion's

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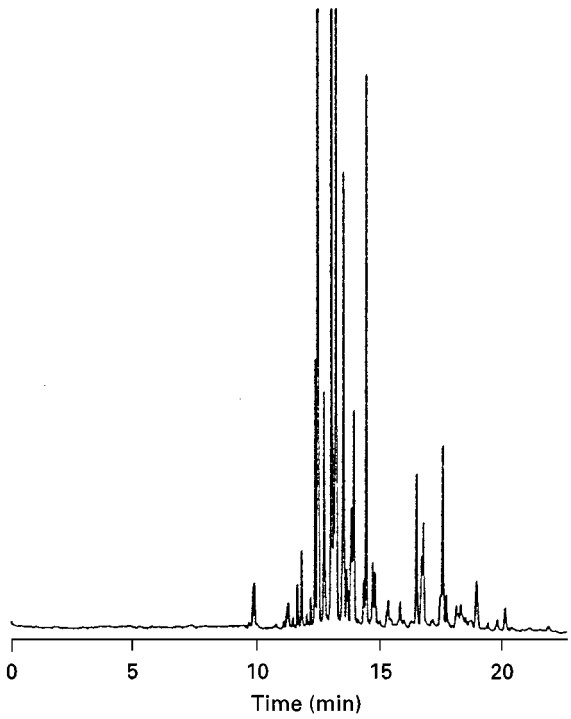

Figure 1 Capillary zone electrophoresis separation of fluorescamine-labelled peptides obtained from a tryptic digest of reduced and carboxymethylated egg white lysozyme. (Adapted with permission from Jorgenson JW and Lukacs KD (1981) Zone electrophoresis in open-tubular glass capillaries: preliminary data on performance. *Journal of High Resolution Chromatography and Chromatographic Communications* 4: 230–231.)

linear velocity per unit of electric field:

$$\mu = \frac{u}{E} = \frac{q}{6\pi r\eta} \qquad [2]$$

From eqn [2], it can be seen that the ion's effective charge, its size and the viscosity of the solution decide ionic mobility. Thus in a given system ionic mobility is an intrinsic property of an ion. Usually ionic mobility cannot be directly derived from eqn [2], as the parameters are not easily accessible quantities. Instead it can be measured based on relevant experimental data, i.e. how long an ion takes to travel through a certain distance under a definite electric field, as follows:

$$\mu = \frac{u}{E} = \frac{L_{\text{eff}}}{t_{\text{m}}} \times \frac{1}{E} = \frac{L_{\text{eff}}}{t_{\text{m}}} \times \frac{1}{V/L_{\text{tot}}} = \frac{L_{\text{eff}} \times L_{\text{tot}}}{V \times t_{\text{m}}} \qquad [3]$$

where L_{eff} and L_{tot} are the effective migration length (from inlet to detection window) and total migration length, respectively, V is the applied voltage, and t_{m} is the migration time of the ion. For a CE system operated under a constant voltage, L_{eff}, L_{tot} and V are all fixed. Hence electrophoretic mobility, the inherent attribute of an ion is directly reflected by its migration time. This provides the theoretical basis of using migration time as a means of identifying an ion in CE.

Electroosmotic Flow (EOF)

Electroosmosis is a fundamental electrokinetic effect involving movement of the bulk solution against a charged solid surface under the influence of an electric field. In the case of CE, the capillary inner wall usually carries negative charges due to the deprotonation of silanol groups. For the part of the liquid adjacent to the capillary wall, build-up of cations takes place to counterbalance the negative charges on the capillary surface. According to Stern's double layer model the solution containing net cations can be divided into two regions, namely a rigid layer and a diffuse double layer. The rigid layer is immediately adjacent to the capillary wall, so the cations within it are largely immobilized owing to the strong electrostatic interaction with the wall. The diffuse layer is slightly away from the wall, hence the cations inside are mobile. Upon the application of a voltage, these cations together with their surrounding hydrating water will migrate towards the cathode. The cohesive nature of water causes the whole solution inside the capillary to be dragged forward, generating a net flow across the capillary. This is named the electroosmotic flow (EOF). The magnitude of the EOF can be described via the Helmultz equation:

$$\mu_{\text{eo}} = \frac{\varepsilon\zeta}{4\pi\eta} \qquad [4]$$

where ε is the dielectric constant of the buffer solution, ξ is the zeta-potential across the diffuse layer, and η is the viscosity of the electrolyte. Unlike conventional electrophoresis where EOF is regarded as unfavourable and thus usually suppressed, in CE it has several important positive implications.

First, the existence of an EOF offers a simple and highly efficient way of driving a separation system. The zeta-potential is uniformly distributed within an extremely narrow cylindrical region along the whole capillary so the bulk electrolyte solution is pumped out of the capillary with virtually no pressure drop (**Figure 2**). A 'plug-like' flow is obtained, which subsequently contributes to high column performance. This is advantageous over the conventional pumping methods such as in HPLC, where the pressure-based flow always introduces a parabolic profile thus adding to the loss of column efficiency.

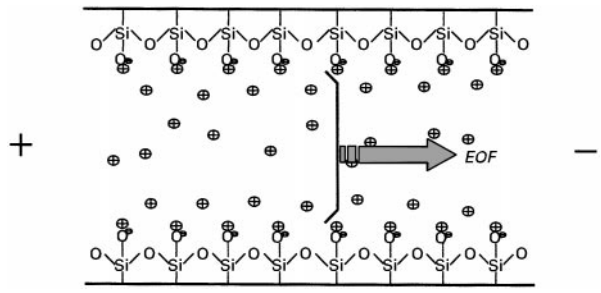

Figure 2 The generation of electroosmotic flow (EOF) in a silica capillary.

Second, the presence of EOF affects the apparent mobilities of ions (**Figure 3**). In any electrophoretic separation system where EOF is not fully suppressed, the observed mobility of a charged species will be the resultant of its effective electrophoretic mobility and EOF:

$$\mu_{obs} = \mu_{ep} + \mu_{eo} \qquad [5]$$

Under normal conditions, with EOF directs towards the cathode, obviously cations will be accelerated, while anions will be decelerated. If the magnitude of the EOF exceeds the mobilities of the anions, the anions will be swept towards the detection side, thus allowing the simultaneous analysis of cationic and anionic species. As the magnitude and direction of EOF will affect how long the analytes stay inside the separation capillary, manipulation of EOF often becomes a core issue for effecting a satisfactory resolution. Since the formation of EOF involves two phases (capillary wall and running buffer), any modification to their chemistries will bring about a change in EOF.

Causes of Band Broadening

As in a chromatographic process, in electrophoresis it is necessary to contain the ionic species within narrow bands while creating sufficient mobility differences. How narrow a band is depends not only on the various dispersive factors inherent to the electrophoretic process, but also on how well the whole process is performed. The common causes of band broadening in CE include longitudinal diffusion, injection-related volume overloading, thermal effects, electrodispersion, wall adsorption, etc. These band broadening mechanisms are deemed to be random and independent events, so that the concept of summation of variances can be used to evaluate the contributions of individual factors to the overall band broadening effect, that is:

$$\sigma_{tot}^2 = \sigma_{diff}^2 + \sigma_{inj}^2 + \sigma_{therm}^2 + \sigma_{wall}^2 + \sigma_{electr}^2 + \sigma_{other}^2 \qquad [6]$$

A brief description of these band broadening factors is given below.

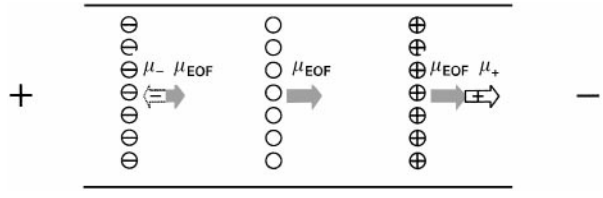

Figure 3 Effect of EOF on the apparent mobilities of anions and cations.

Longitudinal diffusion In the course of electrophoretic transportation of an analyte band along the capillary, the sample molecules will inevitably have a tendency to enter the surrounding buffer solution because of the apparent concentration difference, leading to a wider and more dilute sample band. According to Einstein's diffusion equation, band dispersion due to longitudinal diffusion is a function of diffusion coefficient and time:

$$\sigma_{diff}^2 = 2D_m t \qquad [7]$$

Under an ideal situation, longitudinal diffusion becomes the only unavoidable band broadening process. Therefore it defines the maximum attainable column efficiency in CE. Based on chromatographic theory, the maximum obtainable theoretical plates (N) can be derived as follows:

$$N = \frac{L^2}{\sigma^2} = \frac{L^2}{2D_m t} = \frac{L^2}{2D_m \times (L/v)}$$
$$= \frac{L^2}{2D_m \times [(L_{eff} \times L_{tot})/\mu V]} = \frac{\mu V}{2D_m} \qquad [8]$$

Thus the maximum column efficiency in CE is proportional to the mobility and voltage, while inversely proportional to the diffusion coefficient. Considering that mobilities of ions range between 10^{-4} and 10^{-3} cm^2 V^{-1} s^{-1}, diffusion coefficients from 10^{-7} to 10^{-5} cm^2 s^{-1}, and an applied voltage up to 10^4 V, the attainable theoretical plate number would be in the order of 10^5–10^6, which is much higher than any conventional HPLC approach. Eqn [8] also suggests that in principle CE should be well suited for the separation of high-mass charged particles such as biopolymers, since their diffusion coefficients are extremely low. This has been demonstrated in the most successful resolution of DNA fragments and proteins where plate numbers of 10^6 have been reached. It should be emphasized

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that eqn [8] is only valid under the precondition that longitudinal diffusion plays a predominant role among the various band broadening mechanisms. In other words, to achieve the maximum column efficiency, the electrophoretic separation should be carried out in such a way that all the other potential band dispersions are curbed well below the magnitude of the longitudinal diffusion effect.

Injection related volume overload During sample injection, a finite volume of sample is placed onto the capillary. The length of this starting plug will contribute directly to the final band width. Treating the original band as rectangular in shape, the variance of this plug can be expressed by:

$$\sigma_{\text{inj}}^2 = \left(\frac{l_{\text{inj}}}{12}\right)^2 \qquad [9]$$

where l_{inj} is the initial plug length. As a rule of thumb, loss of efficiency due to any extraneous dispersion factor should be kept within 10% of the maximum theoretical column efficiency. Assuming a moderate plate number in the order of 10^5 as defined by longitudinal diffusion, it can be easily estimated that the acceptable injection length should be a few millimetres. For the commonly employed capillaries with inner diameter between 50 and 75 μm, the above length is equivalent to only a few nanolitres. So it is obvious that CE's high column efficiency will pose very stringent restriction on the sample size. Any attempt to increase the injection volume in an aim to enhance detection sensitivity may result in a significant loss of column efficiency.

Thermal gradient effect An electrophoretic process is always accompanied by certain amount of thermal effects due to the passage of a current through the resistive medium (Joule heating). For a CE system, the electrical power (P) responsible for the generation of heat can be estimated through the following equation:

$$P = V \times I = \frac{V^2}{R} = \frac{V^2 \pi r^2 \lambda c}{L} \qquad [10]$$

where V is the applied voltage, r and L are capillary inner radius and length, respectively, while λ and c are respectively the molar conductivity and the concentration of the electrolyte solution. While heat generation is uniform for the whole electrolyte solution, heat dissipation is apparently not: the nearer the electrolyte is to the capillary wall, the faster is the heat transferred out to the surroundings. Consequently, a temperature gradient is generally present in the radial direction of the capillary, which is equivalent to the superimposition of a parabolic profile to the otherwise plug-like ion boundaries and bulk flow, as any temperature gradient will be translated into viscosity and mobility gradients in the solution. To minimize the influence of thermal effects on the overall column efficiency, it is imperative to limit the heat generation while maximizing the heat dissipation. In this regard, the use of narrow bore capillaries is particularly recommended because it favours the above two aspects simultaneously. According to eqn [10], for a certain CE system, heat generation may also be controlled through balancing the buffer composition and separation potential.

Wall adsorption effect The capillary surface, like most solid surfaces, never behaves in a completely inert manner to foreign compounds. In HPLC, it has been well known that peak anomalies are often the result of some specific interaction (e.g. hydrogen bonding) between the residual silanol groups and analytes. While similar adverse effects cannot be ruled out, in CE the problem is exacerbated by the fact that under a typical operation condition, the silanol groups along the wall are mostly deprotonated to give a negatively charged capillary surface. When an analyte with a positive charge travels along the capillary, the electrostatic force will tend to attract the analytes onto the wall, causing additional band broadening. This is a feature of the analysis of proteins owing to their low diffusion coefficients and multiple charge sites. Significant efforts have been made to tackle this problem, mostly through the suppression of EOF or complete reversal of the charge status of the capillary wall.

Electrodispersion Electrodispersion is the result of Ohm's law during the electrophoretic separation. It may appear in two instances. First, if the conductivity of the injection plug is larger than that of the buffer solution, an isotachophoresis effect will occur to dilute the original band till the conductivity is equal to the surrounding buffer. Second, during the separation, an analyte zone is 'submerged' into the buffer solution. Any mismatch of its mobility with its co-ions in the buffer will render the local electric field different from that in the normal running buffer. If the mobility of the sample ion is larger than that of its co-ions, then there will be a lower electrical field for the analyte zone. Thus any sample ions diffusing out of the zone will experience a higher electric field, and these ions will speed up along the migration direction. This causes the ions at the rear to re-enter the zone, whereas the ions at the front will drift away from the zone. The accumulative effect of such phenomenon is the formation of a tailing band showing a sharp

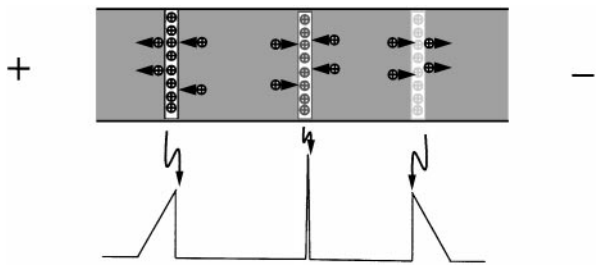

Figure 4 Mobility mismatch-induced band broadening.

trailing boundary but a broadened leading boundary. By a similar argument, if the analyte ion is of lower mobility than the co-ion, a fronting band is expected (**Figure 4**). The higher the sample concentration compared to the buffer concentration, the more pronounced are the nonuniformities with respect to conductivity and field strength, and eventually more severe is the band asymmetry.

From the above discussion it is obvious that, to prevent possible loss of column efficiency due to electrodispersion, the conductivity of the injection plug and the actual sample concentration should be sufficiently low. Theoretical study had shown that, to confine the electrodispersion-related band broadening at a level comparable to longitudinal diffusion, the sample concentration should be two orders below the buffer concentration. To some extent, it is the electrodispersion that limits the mass loadability of a CE system.

Instrumentation

CE can be performed with relatively simple instrumentation as depicted in **Figure 5**. A capillary containing an appropriate separation medium spans two buffer reservoirs, to which the high voltage power supply is connected via the platinum electrodes. Following the introduction of sample at the capillary inlet, a high voltage is applied, thus driving the analytes to travel inside the capillary with different velocities. Somewhere close to the capillary outlet, an on-line detector is installed to monitor the separation process. The resulting signals are fed to the data acquisition device, and finally the result is presented in the form of an electropherogram.

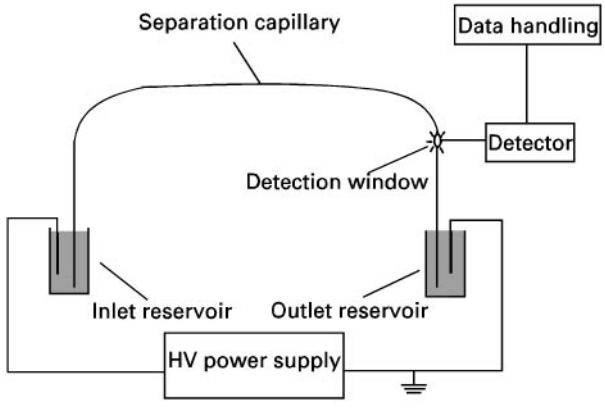

Figure 5 Instrumental setup of a capillary electrophoresis system.

Apart from these fundamental components, commercial CE instruments are commonly equipped with some dedicated facilities, such as an autosampler, pressure regulating unit, capillary thermostatting, and comprehensive supporting software. These added functions allow a sequential analysis of different samples under prespecified conditions, thus ensuring better reproducibility, accuracy and higher throughput. Two modern commercial systems are shown in **Figure 6**.

High Voltage Power Supply

A high voltage power source delivering stable DC potential of $\pm$ 30 kV will satisfy the requirement of most CE applications. Many power supplies offer additional features such as polarity switching, constant potential/current setting, and an interlocked

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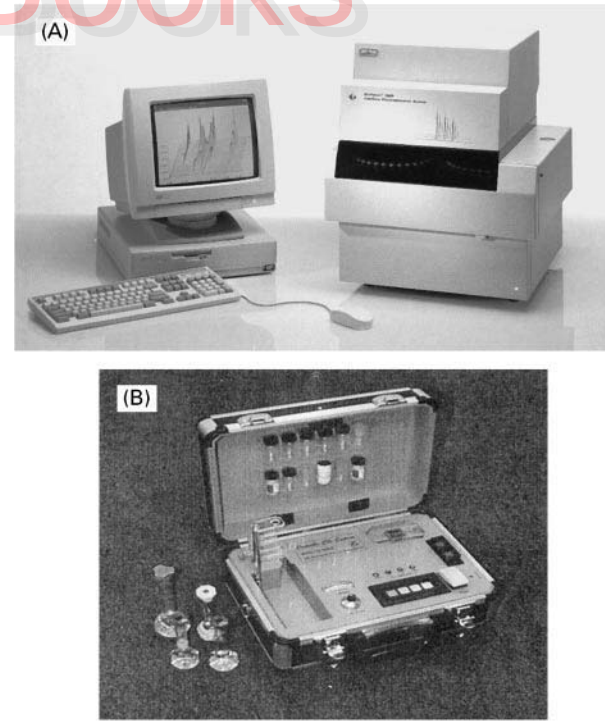

Figure 6 Commercial CE systems. (A) Bench top CE system. Photograph courtesy of Bio-Rad Laboratories. (B) Portable CE system. Photograph courtesy of CE Resources Pte. Ltd.

antielectrical shock loop. In the commercial CE instruments, the power supply is designed with digital communication capability, so that more information such as a current curve can be tracked and retrieved, and the applied voltage can be programmed.

Separation Capillary

Although capillaries made of glass or polymer (e.g. Teflon or Nylon) have found occasional application, fused silica capillaries are used predominantly in CE, largely due to their strength, flexibility and most importantly their excellent UV transparency. Usually the fused silica capillary is coated with a layer of polyimide to enhance its durability. For on-column optical detection a small segment of this coating needs to be removed to provide the detection window. The most commonly used CE capillaries have inner diameter between 20 and 75 μm, outer diameter 100–400 μm and are about 30–100 cm in length.

Sample Injection

To achieve high column efficiency and good quantitative results, sample injection must be performed in a reproducible manner. Since the injection volume in CE is in the nanolitre range, which precludes the use of conventional injection methods, alternative approaches have to be pursued. Hydrodynamic injection and electrokinetic injection have turned out to be the most widely employed sampling techniques.

Hydrodynamic injection introduces a sample based on a pressure difference in the two sides of the capillary. For a laboratory-built instrument, this is realized by simply lifting up the sample vial together with the capillary inlet for a certain period of time (typically a few seconds). The hydrodynamic force will siphon a band of sample solution into the capillary. For commercial instruments, the pressure drop is created by either applying pressure at the inlet side, or imposing a vacuum at the outlet vial. The injection volume can be calculated based on Poiseuille's law:

$$V_{\text{inj}} = \frac{\Delta P \pi r^4 t}{8\eta L} = \frac{\rho g \pi r^4 \Delta h t}{8\eta L} \qquad [11]$$

where ΔP and Δh are the pressure and height differences, respectively, while r, t, η and L represent capillary inner radius, injection time, solution viscosity and capillary total length, respectively.

Electrokinetic injection is based on the transportation of sample ions by electrophoretic movement and EOF. Normally a lower voltage than that for separation purpose is applied for a certain amount of time to allow analytes to migrate into the capillary. The injection volumes of individual components can be calculated through the following equation:

$$V_{\text{inj}} = \frac{(\mu_{\text{i}} + \mu_{\text{eo}})\pi r^2 V t}{L} \qquad [12]$$

where μ_{i}, μ_{eo} are the mobilities of the analyte and EOF, respectively, and V is the injection voltage. For electrokinetic injection the injected amounts of different analytes are dictated by the mobilities of the respective analytes. Thus it is different from hydrodynamic injection, where the sample plug is of entirely the same composition as the original sample solution. To avoid sample injection bias, hydrodynamic injection is preferred. However, in some circumstances hydrodynamic injection may be impracticable (e.g. owing to the high viscosity or low permeability of the separation medium). Electrokinetic injection is then the only viable option, such as in capillary gel electrophoresis. On the other hand, sometimes electrokinetic injection may be exploited in favour of CE operation. For example, it can be employed to diminish the interference of the sample matrix if the components concerned are of low mobilities. Moreover, if the sample is of low conductivity, then sample enrichment during electrokinetic injection is possible by taking advantage of the sample-stacking effect.

Detection

In CE, sample separation is accomplished in an electrolyte solution, so detection strategies analogous to HPLC are adopted. Compared with HPLC, CE column efficiencies are at least one order of magnitude higher, which suggests that solute bands will be narrow and the average concentration of the analyte zone is several times higher than for HPLC. As far as a concentration-sensitive detector concerned, this implies a larger detection signal output. However, so far for CE the concentration sensitivity is usually lower than its HPLC counterparts. This apparent contradiction stems mainly from the small sample size, which poses great difficulties for detection. The fundamental detection schemes in CE fall into three categories: optical (UV absorptive and fluorescent) detection, electrochemical detection, and various hyphenating techniques (typically mass spectrometric detection). The sensitivities of the different detection methods are compared in **Figure 7**.

UV adsorbance This is the most commonly used detection method for CE, mainly because of its simplicity. It is frequently implemented by modifying a HPLC-type UV detector. The normal flow cell is

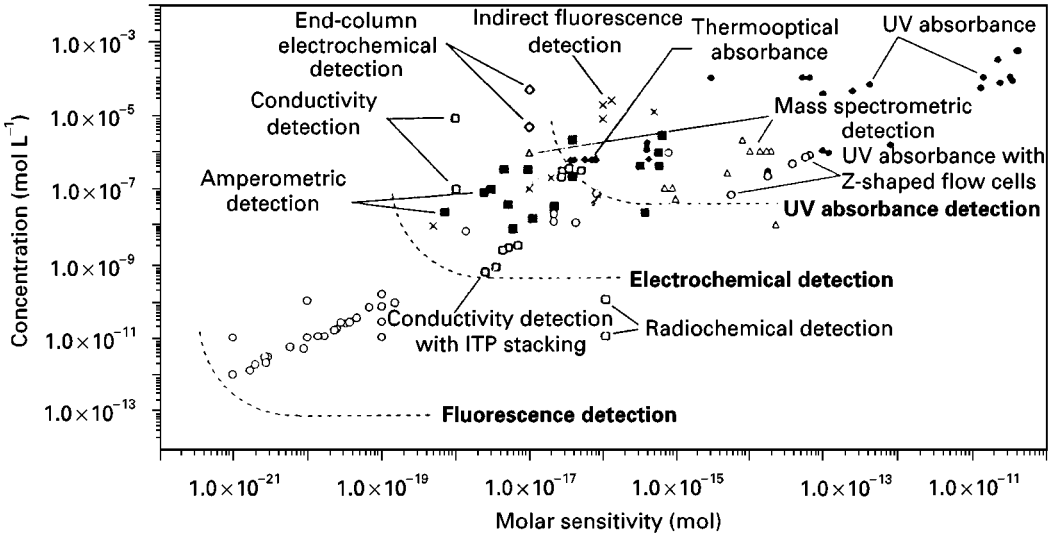

Figure 7 Comparison of sensitivities of various CE detection methods. (Adapted with permission from Landers JP (ed.) *Handbook of Capillary Electrophoresis*, 2nd edn, chap. 10, pp. 425–448. Boca Raton, FL: CRC Press.)

removed and the capillary is mounted directly between the incident lens and photocell, in conjunction with a corresponding aperture. Such on-column detection configuration, though easy to execute, provides only a moderate detection sensitivity with the lower detection limits around 10^{-5} to 10^{-6} mol L^{-1}, as it is associated with two major problems. First, since the capillary is illuminated radially, the maximum optical path is equivalent to the inner diameter of the capillary. This severely reduces the achievable absorbance according to Lambert–Beer's law. Second, due to its unique cylindrical geometry, the capillary tends to act like a lens to defocus the incoming light, thus posing difficulties in light orientation and collection. As a consequence, signal noise and nonlinearity are further aggravated. By bending the capillary into Z-shape or creating a bubble feature on the capillary body, the optical pathlength can be considerably increased. These efforts, coupled with improved detection optics, enhance the detection limits to 10^{-7} mol L^{-1}. The capillary can also be connected to an HPLC-type flow cell (pathlength $\sim$ 1 mm) as in the HP system.

Fluorescence detection Fluorescence detection is so far the most sensitive detection mode available to CE due to the fact that the measurement is performed against a 'dark' background, and fluorescence intensity is less pathlength dependent but directly proportional to excitation power. On-column fluorescent detection can also be realized through adaptation of an HPLC-type detector. Once again, due to the presence of the lens effect of the capillary, improvement of the detection optics is necessary to ensure better focusing of excitation light and more effective collection of emission light. An important development in CE fluorescence detection is the introduction of laser-based excitation sources. Owing to its outstanding coherence, a powerful laser source can be focused into an extremely sharp beam to illuminate the core part of the capillary, thus producing emission light of high intensity. As a result, detection limits of 10^{-9}–10^{-11} mol L^{-1} can be obtained routinely, which is at least two orders of magnitude more sensitive than the conventional approach. Single molecule detection has been demonstrated by fine-tuning the CE system and utilizing laser-induced fluorescence (LIF) detection. A major drawback of fluorescence detection is that the number of analytes with native fluorescence is far less than that with UV absorbance. Therefore, to make use of this highly sensitive detection scheme, derivatization of analytes by tagging with a fluorescent group is often needed.

Indirect photometric detection For compounds without any chrophormore, indirect detection offers a simple, effective way to take advantage of the convenience of on-column optical detection. Here a UV-absorbing or fluorescent compound with the same sign as the sample ions is added to the running buffer to provide a stable background. During the electrophoretic separation, the sample ions will displace a certain amount of background ions due to electroneutrality requirements. Thus the passage of a sample zone through the detection window will appear as a negative peak. In principle, the quantification of a sample in such a way involves differentiating a signal from two large responses, hence the detection

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sensitivity is lower than that for direct photometric detection. To achieve an acceptable detection limit, the background concentration should be kept low. Under such circumstance, any mismatch of mobilities between the sample ions and background co-ions will give rise to a considerable electrodispersion, hence leading to severely distorted peaks. Therefore, selecting a suitable CE background is of special significance for the implementation of indirect optical detection.

Electrochemical detection In electrochemical detection, sample bands are monitored in terms of an electrical signal. Depending on the application, the measured quantity can be conductivity, voltage or current. Accordingly it is referred to as conductimetry, potentiometry or amperometry, respectively. Unlike optical detection in which the measurement output depends strongly upon the available volume, in electrochemical detection the signal relates only to the part of solution that is directly contacting the electrode surfaces. Thus, wherever volume insufficiency precludes the employment of photometric detection, e.g. in case where ultra-narrow bore capillary is used to facilitate fast separation, electrochemical detection may still be a viable choice. Moreover, as long as the analyte is electrochemically active, the detection can be performed directly without involving derivatization, as it may be for photometric detection. Due to these advantages, electrochemical detection gradually gains its popularity in CE practice.

There are two major difficulties involved with implementing electrochemical detection for CE. The first comes from the fact that, it is not easy to make an electrochemical detector function well in the presence of a high voltage, because the separation voltage will produce noise that swamps the minute response of the analytes. Thus, decoupling of the separation electric field from the detection system has to be done to facilitate the measurement. The insulation of the separation voltage can be realized by introducing a fracture or a gap structure in the capillary through which the electrical grounding is made, such that the segment behind the fracture or the gap can be used for the accommodation of the electrochemical electrodes. Alternatively, to shield off the high voltage, the detecting elements may be mounted immediately outside the outlet of the separation capillary. A more elegant way is to etch the capillary end to generate an enlarged conical cuvette from where the detecting electrodes are installed. In this way the interference of high voltage can be eliminated because the electric field decays very rapidly before the enlarged conical part. The second difficulty stems from the need to fabricate microelectrodes and mount them into an extremely small space, usually defined by the separation capillary. While the current state-of-the-art allows the preparation of microelectrodes with dimensions down to several μm, the installation of the microsensing elements to the detection region is a task requiring complicated microfabrication techniques and great patience. All three types of electrochemical detection have shown their feasibility in CE with typical detection limits in the range of 10^{-7}–10^{-9} mol L^{-1}.

Hyphenation with mass spectrometry Following the great success in interfacing HPLC with mass spectrometry, it is a logical move to explore the potential of CE-MS. For a successful implementation of CE-MS, keeping a proper electrical contact at the MS side is essential. This has been achieved through a variety of means, such as contacting via coaxial sheath flow, or through a liquid junction. Sheathless contact has been realized by attaching the metal-coated separation capillary tip directly to the ion source emitter. For sample ionization and transportation, the electrospray ionization interface (ESI) is predominantly adopted, mainly because ESI is operated under almost atmospheric pressure, thus not conflicting with CE separation. Moreover, ESI ionization works with electrostatically induced nebulization, in which compounds are ionized with different charge status depending on their relative molecular masses and shapes. In principle, this allows determination of relative molecular mass for a large range of compounds, from small molecules to large biopolymers (such as proteins and polysaccharides). Thus it is applicable to all types of CE analytes. As for the mass analyser, the high efficiency of CE separation demands a mass analyser with a fast scan rate. The time-of-flight (TOF) mass spectrometer is probably the most promising candidate, but the quadrupole mass spectrometer is currently the workhorse for CE-MS owing to its commercial availability and relatively low cost. Its insufficient scan speed may be compensated partially through the manipulation of the electric field, i.e. when a certain analyte is migrating out of the capillary, the applied voltage can be reduced temporarily, such that the analyte of interest will remain in the ionization chamber for a longer time. With careful optimization of parameters, CE-MS has shown detection limits in the attomole range (10^{-18} mol mass).

Separation Formats of CE

One of the advantages of CE is that many types of separation can be performed with the instrument

described in Figure 2. This is made possible by simply altering the separation medium inside the capillary and utilizing an appropriate buffer system. The commonly employed separation formats in CE can be divided into the following.

Capillary Zone Electrophoresis (CZE)

Among the various CE formats CZE is the simplest and most popular. Here a homogenous free solution is employed to maintain a constant electric field along the capillary. Ionic species are separated inside this supporting solution according to their different charge-mass ratios, thus forming segregate zones. The desired separation selectivity can be achieved by simply optimizing the parameters of the carrier electrolyte particularly pH, as well as ionic strength and the type and concentration of various EOF modifiers. A number of secondary equilibria are available to strengthen the separation selectivity further.

Although CZE is commonly performed with an aqueous buffer, it can also be implemented in a non-aqueous medium by using organic solvents and compatible conductive salts. The effect of replacing water with an organic solvent in CE can be understood from the fact that organic solvents possess significantly different viscosities and dielectric constants compared with water. Thus, changes in the magnitude of EOF and migration behaviours of charged analytes are expected in non-aqueous CE, which can be seen from eqns [2] and [4]. Furthermore, organic solvents differ in their capacity to stabilize equilibria. Thus, in organic media, the charge status of organic analytes can be dramatically different from that in an aqueous medium, hence leading to quite different separation selectivity. Additionally, it is evidenced that organic media are capable of promoting certain mechanisms such as inclusion interaction and ion-pair formation, which enhance possibilities of achieving the desired separation. Moreover, with a water dominated buffer, it is very difficult to conduct CE separations of hydrophobic analytes. Under such circumstances, switching to a non-aqueous buffer system would provide an efficient solution. Finally, it is noted that, when an organic medium is utilized for CZE separations, the electrophoretic current is reduced considerably. As a result, even though capillaries of relatively wide inner diameter are employed, Joule heating is at a manageable level, thus enabling the enhancement of detection sensitivity through the usage of wide bore capillaries. In short, non-aqueous CZE is an attractive means for extending the applicability of CE.

Capillary Gel Electrophoresis (CGE)

For this format, polymeric networks are present along the electrophoretic pathway of analytes, which causes charged species to be resolved on the basis of their physical sizes rather than charge-to-mass ratios. Accordingly this separation process is also commonly known as 'molecular sieving'. It is particularly suitable for the separation of biomacromolecules consisting of numerous repeat charge units, such as DNA fragments, SDS-denatured proteins and polysaccharides (**Figure 8**). CGE may be considered as an adaptation of traditional gel electrophoresis into its capillary format. However, traditional gel electrophoresis use only cross-linked polyacrylamide (so called 'chemical gel'), while for CGE both cross-linked polymer gels and various polymer solutions can be employed to create the sieving pores. The use of polymer solutions instead of chemical gels facilitates renewing of the sieving medium by simply flushing out the original solution, and refilling with another. It also alleviates some technical problems associated with chemical gel such as gel shrinkage, bubble formation and matrix collapse, hence improving the separation reproducibility.

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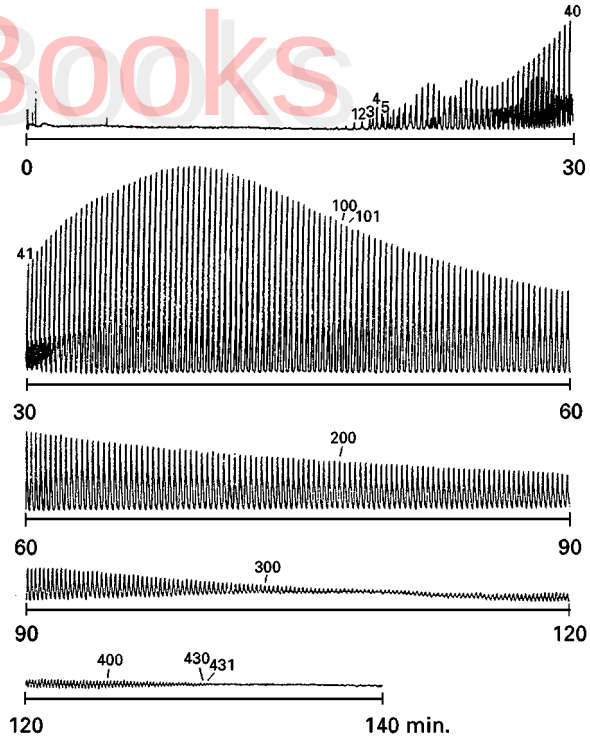

Figure 8 CGE separation of poly(uridine 5′-phosphate). (Adapted with permission from Yin HF, Lux JA and Schomburg G (1990) Production of polyacrylamide gel filled capillaries for capillary gel electrophoresis (CGE): influence of capillary surface pretreatment on performace and stability. *Journal of High Resolution Chromatography* 15: 624–627.)

Capillary Isotachophoresis (CITP)

In CITP, a leading electrolyte of higher mobility than any of the sample components is filled into the separation capillary and outlet reservoir, while a terminating electrolyte of lower mobility than any of the sample components is placed in the inlet reservoir. The sample is applied from the capillary inlet. This arrangement ensures that there will be no mixing of sample with the two electrolytes during the whole electrophoretic process. As a consequence, upon application of an electric field, the main change happens inside the sample band itself, i.e. the sample components tend to 'queue up' according to their effective mobility orders. These components are relocated with corresponding changes in their concentrations and band lengths. Ultimately a series of consecutive 'pure zones' containing only the individual substances are formed. This is the steady state as there will be no more changes for the sample except that all the zones will continue to migrate out of the capillary with an identical velocity (hence the term 'isotachophoresis'). Unlike the other types of CE separation, in CITP the concentration of a specific zone is predetermined by the concentration of the leading electrolyte and the relative mobility of the ion of interest with respect to the leading electrolyte, while the sample amount is reflected by the zone length. Due to the unique stepwise increase in electric field starting from the leading electrolyte side, which prevents a component from drifting off its own band, sharp boundaries can be maintained more readily. Therefore CITP can be implemented using tubes of relatively large i.d. (e.g. 0.2–0.8 mm). Nevertheless, CITP as an analytical tool has largely lost its popularity since the advent of CZE, probably due to the fact that a thorough knowledge of the sample is required before the separation. Furthermore, the isotachopherogram is usually step-shaped, and the steps are not time related, thus rendering automatic identification and quantification difficult. CITP is often applied as a sample pre-concentration step before CZE separations to enhance detection sensitivity.

Capillary Isoelectric Focusing (CIEF)

This is another example of an adaptation of a conventional electrophoresis principle to a capillary format. CIEF exploits differences in isoelectric points (p*I*s), a unique characteristic of amphoteric compounds under which its acidic and basic groups dissociate to an equal extent, so that the whole molecule exhibits no net charge. It is only suitable for the separation of compounds like amino acids, peptides and proteins. The operation of CIEF relies on a mixture of carrier ampholytes with isoelectric points in a certain range and in close proximity to each other. Such a mixture is commonly formed by a series of synthetic polyamino polycarboxylic acids. The capillary is filled with the carrier ampholyte solution and a small amount of sample, and dipped into the buffer reservoirs that contain acid and base, respectively. Under the electric field, different ampholytes will migrate along the capillary until they reach positions corresponding to their p*I* values, where they stand still. Collectively a stable pH gradient is formed across the capillary. The sample components, too, will migrate until they find positions equivalent to their p*I* values. When such a steady state is obtained, an immobilization step (by utilizing electrophoretic movement or pressure) is performed, and the separated analyte bands will be forced out and passed through the detector. If the carrier ampholyte mixture is well prepared and a sufficient time is allowed for the electric focusing process, a very high column efficiency can be realized.

Micellar Electrokinetic Chromatography (MEKC)

Neutral species can never be resolved with traditional electrophoretic techniques since they have no electrophoretic mobility. After the birth of CE, this problem was solved by Terabe *et al*. through the addition of a charged surfactant to the running buffer. Typically a negatively charged surfactant is added at a concentration above its critical micelle concentration (CMC). Under such circumstances, micelles are formed, which allow neutral compounds to be retained based on their hydrophobicities. Under an electrical field, the negatively charged micelles move toward the anode side, while a strong EOF moves toward the cathode side (**Figure 9**). As EOF usually exceeds the electrophoretic mobility of micelles, the micelles will eventually be swept out in the same

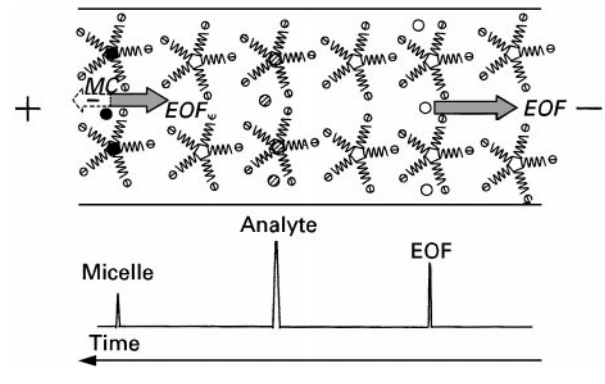

Figure 9 Separation mechanism of MEKC.

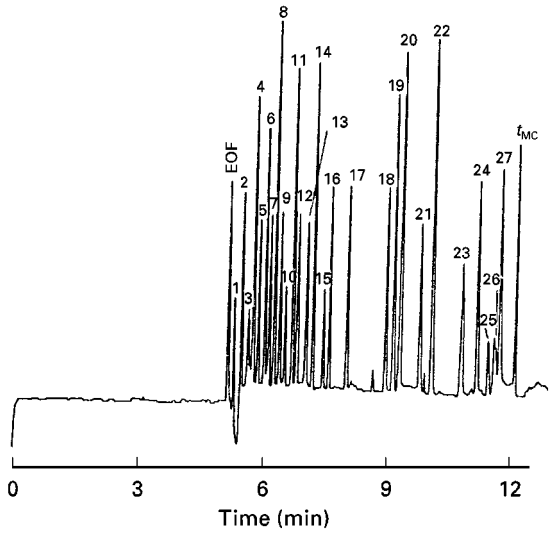

Figure 10 Separation of small neutral aromatic compounds by bile salt-based MEKC. (Unpublished results.)

direction. Thus a migration time window is defined within which the neutral compounds can be separated according to their affinities for the micelle microenvironment. If a positively charged surfactant is used, then similar resolution of neutral analytes can again be obtained, the only difference being that the EOF will be reversed owing to the adsorption of cationic surfactant molecules onto the capillary. The polarity of the separation voltage, therefore, needs to be reversed. The micelle-forming reagents for MEKC are not limited to synthetic detergents – any chemical with similar surface activity can be employed. These include biogenic surfactants such as bile salts (**Figure 10**), and as some synthetic high mass-to-charge polymers.

Affinity Capillary Electrophoresis (ACE)

Compared with other types of CE techniques, ACE represents a relatively recent development. Introduced in the early 1990s, ACE is performed on the basis of specific or non-specific affinity interactions between receptor and ligand molecules, typically biomolecules. Theoretically, if either receptor or ligand is a charge species, their binding complex would show a different electrophoretic mobility from that of the parent molecule due to the changes in charge-mass ratio. Thus, measuring the changes in electrophoretic mobility of the receptor via CZE mode provides an excellent way to study aspects of the receptor–ligand binding.

Depending on the binding strength of the receptor–ligand pair and the operational procedure, ACE can be divided into three different categories, namely, non-equilibrium mode, dynamic equilibrium mode and immobilizing mode. Equilibrium ACE is well suited to studying strong binding systems, in which the sample is injected as an equilibrated mixture of receptor and ligand, whereas the electrophoresis medium contains only the supporting buffer. In such cases, CE serves merely as a tool to separate and determine the free and bound receptor molecules. Dynamic equilibrium ACE is typically employed for weak to moderate binding system, in which the receptor is injected as the sample, while ligand of varied concentration is incorporated in the running buffer. In this case, free and bound molecules are not separated due to the fast on-and-off kinetics, but rather, they are detected as single peaks. The immobilizing mode is self-explanatory, for which ligand is attached to the capillary wall, or more commonly, onto a supporting material via an appropriate bonding chemistry, while sample (receptor) is driven over the active surface by application of an electric field. Again, the migration behaviour of the receptor is a good indication of receptor–ligand interaction strength.

ACE has become a powerful tool in diverse fields, including the measurement of binding constants, the study of binding kinetics, the determination of interaction stoichiometry, the characterization of biomolecules and the separation of enantiomers, etc. ACE has been applied to the investigation of a number of biologically important systems, such as the interaction of polypeptides with immunoglobulins, polypeptides, carbohydrates, nucleic acids, drugs, etc.

ACE bears some resemblance to classical gel affinity electrophoresis and conventional affinity chromatography, in that they all utilize specific interactions to effect separations. However, ACE inherently has an unsurpassed advantages, that is, the buffer conditions (ionic strength and pH) can be finely tuned to give a perfect mimic of the real physiological environment, so that more precise characterisation of many binding processes is possible, provided the wall adsorption problem can be addressed properly.

Applications

The introduction of CE has resulted in a dramatic expansion of the applicability of electrophoresis as a separation tool. While conventional slab gel electrophoresis is mainly limited to the separation of biosubstances such as proteins and DNA fragments, CE has been utilized to resolve a much broader spectrum of substances, ranging from simple ions

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through small molecules to macromolecules. High mass biomolecules such as proteins, DNA and polysaccharides can be separated by CZE as well as CGE. Smaller charged molecules such as amino acids, peptides, organic acids and amines can be resolved via CZE. Simple ions, both cations and anions, can be easily separated via CZE with conductivity detection. CZE separations of ions with indirect UV detection, dubbed as capillary ion analysis (CIA) has been accepted rapidly as an alternative to ion chromatography owing to its simplicity, speed and low cost. The separation of various neutral compounds has been made possible through MEKC or CEC. Chiral separations, an area hardly touched by conventional electrophoresis, is increasingly carried out by CE. Many chiral selectors (cyclodextrin derivatives, amino acids, proteins, optical micelles, etc.) may be added to the buffer solution to induce enantiodis crimination based on one of several mechanisms (host–guest complexation, ligand exchange and solubilization by micelles, etc.). The unequal stability of dynamically formed diastereoisomers cause the optical isomers to be moved out of the capillary with different velocities, and enantioseparation is achieved. Because of its far-reaching capabilities, CE is becoming ubiquitous in almost all analytical fields.

Future Prospects

The introduction of CE in the early 1980s has had a huge impact on numerous scientific fields. It can be anticipated that in the future CE will continue to evolve into a fully fledged analytical technique that will benefit many research disciplines. Based on the characteristics of CE and its current status, several directions deserving special attention can be envisaged.

CE is hailed for its high column efficiency and outstanding mass sensitivity. The concentration sensitivity of CE remains relatively low compared to HPLC, thus limiting its applications in areas such as trace impurity determination and environmental analysis. Therefore, improving the detection sensitivity will continue to be a topic for development. Creative detection configurations, interplay from micromechanic and microelectronics, together with advancement of light sources, may remarkably enhance the sensitivity of optical detection. To facilitate the use of electrochemical detection, efforts must be made to provide better microelectrodes with reasonable ruggedness. With the enhanced performance and reduced cost, a more common use of sophisticated hyphenation techniques such as CE-MS is expected.

On the other hand, the ability of CE to handle extremely small sample quantities is attractive for the direct probing of micro-entities. The recently emerged single cell analysis is a good example of this. With further improvements in sampling techniques and detection schemes, it is believed that in the near future, CE-based methodologies will allow us to investigate the chemistries of a wider spectrum of cells, thus enriching our knowledge about many biological processes essential to life.

Finally, due to its instrumental simplicity, CE is amenable to further miniaturization. CE on a glass chip has been successfully demonstrated by borrowing microfabrication concepts from the microelectronics industry. These devices feature the integration of injection, separation and detection, as well as sophisticated designs with intricate patterns and multi-channel arrays, thus producing an unprecedented analytical platform which is fully manipulated by applying voltages. As the plate format possesses an excellent ability for heat dissipation, electric fields up to 2000 $V\,cm^{-1}$ may be applied across the separation channels, hence shortening the analytical time scale to minutes or even seconds. With continuous maturity of relevant technologies, such an ultra-high speed separation method may provide a solution to some formidable tasks including DNA sequencing.

See also: **II/Electrophoresis:** Electrochromatography Thin Layer.

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